National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 968c

Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum

Standard Reference Material (SRM) 968c is intended for use in validating methods for determining fat-soluble vitamins, carotenoids, and cholesterol in human serum and plasma. This SRM can also be used for quality assurance when assigning values to in-house control materials for these constituents. A unit of SRM 968c consists of two vials of lyophilized human serum, one vial at each of two different concentration levels. The lyophilized serum in each vial must be reconstituted with 1.00 mL of HPLC-grade water before use.

Certified Concentration Values: The certified concentration values of *trans*-retinol, δ-tocopherol, γ-tocopherol, α-tocopherol, *trans*-β-carotene, total β-carotene, and cholesterol in reconstituted SRM 968c are provided in Table 1. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or accounted for by NIST [1]. The certified values for cholesterol were determined from measurements using the NIST definitive method, gas chromatography-isotope dilution mass spectrometry (GC-IDMS). The certified concentration values for the fat-soluble vitamins and carotenoids are based on the agreement of results from two or more different liquid chromatography (LC) procedures performed at NIST and the median of results from an interlaboratory comparison exercise among institutions that participate in the NIST Micronutrients Measurement Quality Assurance Program. An alphabetized listing of these institutions is provided in Appendix A.

Reference Concentration Values: Reference concentration values for *trans*-lutein, total lutein, total zeaxanthin, total β-cryptoxanthin, *trans*-α-carotene, total α-carotene, *trans*-lycopene, and total lycopene are provided in Table 2. Reference values are noncertified values that are the best estimate of the true values based on available data; however, the values do not meet the NIST criteria for certification and are provided with associated uncertainties that may reflect only measurement precision, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods. The reference values for the carotenoids are based on the agreement of results from analytical methods performed at NIST and the median of results from an interlaboratory comparison exercise. Values for some carotenoids are designated as reference values because the identity of components present in the measured chromatographic peak is less certain. The reference values for 25-hydroxyvitamin D are based on the agreement of results from six collaborating laboratories using three different methods.

Information Concentration Values: Information values for 25-hydroxyvitamin D_2, 25-hydroxyvitamin D_3, *trans*-2',3'-anhydrolutein, total α-cryptoxanthin, total *cis*-β-cryptoxanthin, phytofluene, and retinyl palmitate are provided in Table 3. An information value is considered to be a value that may be of interest to the SRM user, but insufficient information is available to assess the uncertainty associated with the value or only a limited number of analyses were performed.

Expiration of Certification: The certified values of this SRM lot are valid until **30 September 2006**, within the measurement uncertainties specified, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see Instructions for Use). However, the certification is nullified if the SRM is damaged, contaminated, or modified.

Maintenance of SRM Certificate: NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Return of the attached registration card will facilitate notification.

The support aspects involved with the certification and issuance of this SRM were coordinated through the NIST Standard Reference Materials Program by J.C. Colbert.

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The overall direction and coordination of the preparation and analytical measurements leading to the certification of this SRM were performed by J. Brown Thomas, L.C. Sander, M.J. Welch, and S.A. Wise of the NIST Analytical Chemistry Division.

Preparation of SRM 968c and analytical measurements at NIST were performed by J. Brown Thomas, K.E. Sharpless, and L.T. Sniegoski of the NIST Analytical Chemistry Division and M.C. Kline and J.W. Redman of the NIST Biotechnology Division. Collaborating laboratories that performed analytical measurements are listed in Appendix A.

Statistical consultation was provided by L.M. Gill and J.H. Yen of the NIST Statistical Engineering Division and D.L. Duewer of the NIST Analytical Chemistry Division.

NOTICE AND WARNING TO USERS

Warning: SRM 968c IS INTENDED FOR IN-VITRO DIAGNOSTIC USE ONLY. THIS IS A HUMAN-SOURCE MATERIAL. HANDLE PRODUCT AS A BIOHAZARDOUS MATERIAL CAPABLE OF TRANSMITTING INFECTIOUS DISEASE. The supplier of the source materials used to prepare this product found the materials to be non-reactive when tested for Hepatitis B surface antigen (HBsAg), human immunodeficiency virus (HIV), Hepatitis C virus (HCV), and human immunodeficiency virus 1 antigen (HIV-1Ag) by Food and Drug Administration (FDA) licensed tests. However, because no test method can offer complete assurance that HIV, hepatitis viruses, or other infectious agents are absent, this SRM should be handled at the Biosafety Level 2 for any potentially infectious human serum or blood specimen [2].

Storage: Until required for use, SRM 968c should be stored in the dark at a temperature between -20 ºC and -80 ºC. If carotenoids are to be measured, the unit should be stored at or below -70 ºC in the dark. Carotenoids appear to be less stable than the retinoids and the tocopherols at -20 ºC [3-6].

Instructions for Use: SRM 968c is provided as a set of two vials of lyophilized serum that must be reconstituted prior to use. Before reconstitution, the vials should be allowed to stand at room temperature for at least 30 min under subdued light. At room temperature, the vials appear to be at slight positive pressure; therefore, the vials may be vented by insertion of an empty syringe needle. To achieve the certified concentrations, the freeze-dried serum must be reconstituted with 1.00 mL of HPLC-grade water. Dissolution should be facilitated by ultrasonic agitation for 3 min to 5 min or by intermittent swirling for at least 15 min. Vigorous shaking or mechanical swirling should be avoided as it may cause the formation of foam. After reconstitution, the contents should be used immediately or stored at -20 ºC for no more than three days with minimal (fewer than five) freeze/thaw cycles [6]. Precautions should be taken to avoid exposure to strong UV light and direct sunlight.

PREPARATION AND ANALYSIS[1]

Preparation of SRM Serum Pools: Both levels of SRM 968c were prepared from source plasma units (3 L) obtained from Interstate Blood Bank, Inc., Memphis, TN. All units were tested and found negative for HBsAg, HIV, HCV, and HIV-1Ag prior to shipment to NIST. Units were stored at -80 ºC until use. Plasma units for each level were selected to create the desired naturally occurring level of ß-carotene. Level I also contained 400 mL defibrinated/delipidized human serum (Western States Plasma Company, Fallbrook, CA), which was also tested and found negative for HBsAg, HIV, HCV, and HIV-1Ag prior to shipment to NIST. Selected units were thawed in a 25 ºC water bath and then filtered under vacuum and reduced light (yellow light). The combined units for Level I were then spiked with ethanol solutions of *trans*-retinol and γ-tocopherol. The concentrations of the spiking solutions were based on target values relative to the physiological levels of the analytes in human serum and were determined by spectrophotometry. The purity of the solutions was determined by LC at the wavelength of maximum absorption. After spiking, the material was mixed for 30 min at ambient temperature, under argon, then frozen at -20 ºC. On the day the material was aliquoted for freeze-drying, it was thawed in a 25 ºC water bath and filtered as described above.

[1] Certain commercial products are identified in this certificate to adequately describe the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the products identified are necessarily the best available for this purpose.

Level II was prepared in a similar manner and was spiked with α-tocopherol and ð-tocopherol. Ethanol spiking solutions for these two analytes were prepared and analyzed as described above.

Two freeze-drying runs per level were required. Each level was thawed at 25 ºC and filtered as previously described. Aliquots (1 mL each) of serum from each unit were dispensed into 2-mL amber vials using a calibrated air-displacement pipettor. Vials were labeled and randomly placed on drying trays. The average time in the freeze dryer was 76 h for all samples. Prior to removal from the freeze-drying chamber, the vials were sealed under slight vacuum with argon (at approximately 15 ºC). After removal from the freeze dryer, vials were stored at -80 ºC. Vials for certification analyses were selected using a stratified random sampling scheme.

Description of Analytical Measurements Used for Value Assignment

A. Measurement of the Fat-Soluble Vitamins and Carotenoids in SRM 968c

The assigned values for selected fat-soluble vitamins and carotenoids in this SRM were derived from results of analyses performed by NIST and 53 collaborating institutions (listed in Appendix A). Because the maintenance of pure and stable primary reference compounds for these analytes is difficult, detector responses were calibrated against solutions whose concentrations were determined by spectrophotometry with corrections made for purity as determined by LC. NIST analyses were based on the absorptivities provided in Figure 1. Three different LC techniques (chromatograms shown in Figure 2) were used at NIST for the determination of the fat-soluble vitamins and carotenoids in the SRM [7-10]. Details of the three LC methods are provided below. (**NOTE:** The compositions of the solvent mixtures described in these methods are expressed as volume fractions in percent [11].) SRM 968b Fat-Soluble Vitamins and Cholesterol in Human Serum was run for quality control at NIST during the certification analyses of SRM 968c.

Preparation of Serum Extract for LC Analysis

Lyophilized samples were equilibrated at room temperature for at least 30 min and reconstituted with 1.00 mL of HPLC-grade water. Dissolution of the samples was ensured by ultrasonic agitation for at least 3 min and vortex mixing for 10 s. Aliquots of serum (200 µL) were pipetted into glass test tubes and were combined with equal volumes of ethanol containing the internal standard(s) and butylated hydroxytoluene (BHT). The samples were vortex-mixed for 15 s. One mL of hexane was added to each and the samples were vortex-mixed for at least 45 s. The samples were then centrifuged for about 1 min and supernatants were removed and placed in glass vials. This extraction process was repeated and the supernatants were removed and combined with those of the first extraction. The combined extracts were evaporated under a stream of nitrogen under subdued fluorescent light to minimize possible degradation of the analytes, and were reconstituted in either 100 µL ethanol or 50/50 ethyl acetate/ethanol containing 30 mg/L BHT, depending on the LC procedure used. The reconstituted extracts were then vortex-mixed and ultrasonically agitated for approximately 30 s to ensure dissolution and placed in glass inserts in autosampler vials.

Reversed-Phase LC Using a Polymeric C_{18} Stationary Phase

Trans-retinol, δ-tocopherol, γ-tocopherol, α-tocopherol, total lutein, total zeaxanthin, total β-cryptoxanthin, total α-carotene, and *trans*- and total β-carotene were measured in two extracts from each of five vials from each of the two levels of SRM 968c over three days using a 5-µm polymeric [12] C_{18} column (4.6 x 250 mm; Vydac 201TP; Separations Group, Hesperia, CA, USA). A ternary solvent mixture was used in this method. Solvent A was 60 % methanol/10 % butanol/30 % water. Solvent B was 89.5 % methanol/10 % butanol/0.5 % water. An initial 5 min isocratic hold of 75 % solvent A followed by a 40-min linear gradient from 75 % solvent A to 90 % solvent B was used to sequentially determine these analytes in the SRM. UV/visible absorbance detection using a deuterium lamp at the following wavelengths was used: 325 nm for retinol, 292 nm for the tocopherols, and 452 nm for the carotenoids. Tocol (the internal standard) was monitored at 292 nm. A typical separation is shown in Figure 2A.

Reversed-Phase LC Using a C_{18} Stationary Phase with Different Selectivity

Trans-retinol, δ-tocopherol, γ-tocopherol, α-tocopherol, total β-cryptoxanthin, total lycopene, *trans*- and total α-carotene, and *trans*- and total β-carotene were measured in two extracts from each of 17 vials of each level of the SRM over six days using a Bakerbond™ C_{18} column (4.6 x 250 mm; J.T. Baker, Phillipsburg, NJ, USA). This

column exhibits selectivity intermediate to monomeric and polymeric C_{18} columns [12]. This method was also used to assess the homogeneity of the SRM material. A ternary solvent method was used to isolate the analytes from the serum extract. Solvent A was acetonitrile, solvent B was methanol containing 0.05 mol/L ammonium acetate, and solvent C was ethyl acetate. Each of the three solvents contained a volume fraction of 0.05 % triethylamine (TEA) to enhance carotenoid recovery [8]. The method consisted of two linear gradients and an isocratic component. The first gradient ran from 98 % solvent A/2 % solvent B to 75 % solvent A/18 % solvent B/7 % solvent C in 10 min. A second linear gradient ran from this composition to 68 % solvent A/25 % solvent B/7 % solvent C in 5 min. The final composition was held for 15 min longer. The system was then returned to initial conditions of 98 % solvent A/2 % solvent B over 5 min and re-equilibrated for 5 min. In this method, a programmable UV/visible absorbance detector with a tungsten lamp was used for measurement of retinol and the carotenoids at 325 nm and 450 nm, respectively. *Trans*-β-apo-10'-carotenal oxime [13,14] was used as the internal standard for the quantification of retinol and the carotenoids. A fluorescence spectrometer was used to measure the tocopherols and tocol (the internal standard) using an excitation wavelength of 295 nm and an emission wavelength of 335 nm. Signals from both detectors were recorded simultaneously. Typical separations with absorbance and fluorescence detection are shown in Figures 2B and 2C, respectively.

Reversed-Phase LC Using a Polymeric C_{30} Stationary Phase

Measurement of *trans*-lutein, total zeaxanthin, total β-cryptoxanthin, *trans*- and total lycopene, *trans*-α-carotene, and *trans*-β-carotene was provided using a NIST-engineered polymeric C_{30} carotenoid column [15,16]. In this method, two extracts from each of five vials of each level of the SRM were analyzed over three days. The method consisted of two linear gradients and an isocratic component. Solvent A was 8 % water/92 % methanol containing 0.05 mol/L ammonium acetate and a volume fraction of 0.05 % TEA; solvent B was methyl *tert*-butyl ether. The initial solvent composition of the first gradient was 83 % solvent A/17 % solvent B and ran to 59 % solvent A/41% solvent B in 29 min. The second linear gradient ran from this composition to 30 % solvent A/70 % solvent B in 5 min. The final composition was held for 11 min longer to allow the elution of lycopene, then the system was returned to initial conditions (83 % solvent A/17 % solvent B) over 5 min and re-equilibrated for 5 min. A programmable UV/visible absorbance detector with a tungsten lamp was used for measurement of the carotenoids at 450 nm. *Trans*-β-apo-10'-carotenal oxime was used as the internal standard [13,14]. A typical separation is shown in Figure 2D.

Interlaboratory Methods Used for the Analysis of SRM 968c

Retinol, tocopherols, and carotenoids in SRM 968c were measured by collaborating institutions that participated in an interlaboratory comparison exercise in which blind samples of the SRM were distributed as part of the NIST Micronutrients Measurement Quality Assurance Program. Analyses typically involved precipitation of serum proteins with ethanol followed by extraction of the supernatant with a lipophilic solvent (e.g., hexane or petroleum ether). The extracts were then analyzed by LC using various stationary phase and mobile phase combinations, detectors, and internal standards.

Samples were distributed by NIST to six laboratories (identified in Appendix A) for vitamin D analysis. Three different methods of analysis were used. Four laboratories used the DiaSorin® (DiaSorin, Inc., Stillwater, MN, USA) radioimmunoassay procedure for the quantitative determination of 25-hydroxyvitamin D. One laboratory used silicic acid chromatography followed by a competitive protein binding assay, and one laboratory used LC with UV-absorbance detection.

B. Measurement of Cholesterol in SRM 968c

Cholesterol concentrations were determined using the NIST GC-IDMS definitive method [17,18]. Three sets of samples, each consisting of three vials from each of the two levels of the SRM, were analyzed. Each vial was reconstituted with 1.00 mL of HPLC-grade water. An aliquot was subsequently analyzed using a previously established procedure that employs hydrolysis of cholesterol esters using potassium hydroxide in ethanol, followed by extraction with hexane, and derivatization of cholesterol using *bis*(trimethylsilyl)acetamide [18]. Cholesterol-25,26,27-$^{13}C_3$ was used as the internal standard. Duplicate injections of each sample and each standard were made in each set. Quantitation of cholesterol was achieved by the use of a standard curve obtained by measurement of standards of weighed mixtures of SRM 911b Cholesterol and cholesterol-25,26,27-$^{13}C_3$. Two vials of SRM 1951a Lipids in Frozen Human Serum were run as controls in each set.

Description of Calculations Used in Value Assignment

A. Summary Statistics for Analytical Methods

The sets of results for each analyte in both levels of the SRM provided by the three NIST methods were characterized as independent normal distributions. A mean was calculated for each of the three NIST data sets. The expected variability in the mean value is characterized as the standard error of the mean, $SD/\sqrt{n}$, where n is the total number of measurements in the set, and SD is the sample standard deviation. The standard deviations of the means were combined to obtain the combined standard uncertainty, u_c. The combined standard uncertainty is calculated according to the ISO Guide [1] and is intended to represent the measurement error at the level of one standard deviation.

Summary results from an interlaboratory comparison exercise were used to help assign certified or reference values for analytes reported by at least 12 participants. The median of the reported results for a given analyte in a given sample provides a robust estimate of the expected value. The estimate of the standard error of the median is provided by $(0.741 \times IQR)(1.2533 / \sqrt{n})$, where IQR is the inter-quartile range (the range of the central 50 % of the data) and n is the number of participants reporting quantitative values for the particular analyte [19].

B. Certified Values and Their Uncertainties

Retinol, Tocopherols, and ß-Carotene

The certified concentration values for *trans*-retinol, γ-tocopherol, α-tocopherol, *trans*-β-carotene, and total β-carotene are equally weighted means of the medians from the interlaboratory comparison exercise and the means from at least two of the three NIST LC methods. Since NIST analyses found no quantifiable concentrations of *cis*-retinol isomers in either SRM level, the results for "retinol" from the interlaboratory comparison exercise was combined with the NIST results for *trans*-retinol. The certified value for δ-tocopherol is the equally weighted mean from two NIST LC methods.

The expanded uncertainty in the certified values is calculated as $U = ku_c + B$, where k is the coverage factor and B is a bias adjustment for the difference among methods. The coverage factor is determined from the Student's t-distribution corresponding to the appropriate associated degrees of freedom and 95 % confidence for each analyte. The bias adjustment is calculated as the maximum difference between the certified value and the expected values for the individual methods [20]. The true value of the analyte concentration is, with 95 % confidence, expected to be in the interval defined by the certified value ± the expanded uncertainty.

Cholesterol

The certified concentration value for cholesterol in each of the two SRM levels is the mean of the results obtained with the definitive NIST method using GC-IDMS. The dominant source of measurement uncertainty with this method is uncertainty in the measurement of the volume of water used to reconstitute the lyophilized serum. The observed expanded uncertainty in the certified values, calculated as $U = ku_c$, was increased to allow for reconstitution volume uncertainty. The true value of each cholesterol concentration is, with 95 % confidence, expected to be in the interval defined by the certified value ± the expanded uncertainty.

C. Reference Values and Their Uncertainties

Carotenoids (Other than trans-Lutein)

The reference concentration values for total lutein, total zeaxanthin, total β-cryptoxanthin, total lycopene, *trans*-lycopene, and total α-carotene are equally weighted means of the medians from the interlaboratory comparison exercise and the means from at least one of the three NIST LC methods. The reference value for *trans*-α-carotene is the equally weighted mean from two NIST LC methods. No *cis*-α-carotene isomers were detected in the SRM Level I material by either method employed at NIST; the *trans*- and total α-carotene reference values, therefore, represent the same sets of analytical results. The expanded uncertainty for the reference values is calculated as $U = ku_c + B$. The true value of the analyte concentration is, with 95 % confidence, expected to be in the interval defined by the reference value ± the expanded uncertainty.

trans-Lutein

The reference concentration value for *trans*-lutein in each of the two SRM levels is the mean of the NIST analyses using the C_{30} stationary phase. The expanded uncertainty for the reference value of each SRM level is estimated from the relative expanded uncertainty for total lutein. The true value of each *trans*-lutein concentration is, with 95 % confidence, expected to be in the interval defined by the reference value ± the expanded uncertainty.

25-Hydroxyvitamin D

The reference concentration value for 25-hydroxyvitamin D in each of the two SRM levels is the mean of results from the analysis of two vials each by six collaborating laboratories. The expanded uncertainty for these reference values is calculated as $U = ku_c + B$, with k appropriate for n = 12. The true value of each 25-hydroxyvitamin D concentration is, with 95 % confidence, expected to be in the interval defined by the reference value ± the expanded uncertainty.

D. Information Values

Information concentration values are noncertified values with no associated uncertainties because there is insufficient information to assess uncertainties or because only a limited number of analyses were performed. The information concentration values for 25-hydroxyvitamin D_2, 25-hydroxyvitamin D_3, *trans*-2′,3′-anhydrolutein, total *cis*-ß-cryptoxanthin, and phytofluene were derived from results submitted by only one laboratory. The information value for total α-cryptoxanthin was derived from results reported by six collaborating laboratories. The concentration value for retinyl palmitate is the median of results reported by 13 laboratories for Level I of the SRM and 15 laboratories for Level II.

Table 1. Certified Concentration Values for Fat-Soluble Vitamins, β-Carotene, and Cholesterol in SRM 968c[a]

	Level I		Level II	
	µg/mL	µmol/L	µg/mL	µmol/L
trans-Retinol	0.841 ± 0.027	2.93 ± 0.094	0.484 ⟨ 0.012	1.69 ⟨ 0.041
ð-Tocopherol	0.131 ± 0.018	0.325 ± 0.045	0.527 ⟨ 0.071	1.31 ⟨ 0.18
γ-tocopherol[b]	3.90 ± 0.13	9.36 ± 0.30	1.56 ⟨ 0.10	3.73 ⟨ 0.22
α-Tocopherol	7.47 ± 0.47	17.3 ± 1.1	16.79 ⟨ 0.76	39.0 ⟨ 1.8
trans-ß-Carotene	0.157 ± 0.016	0.293 ± 0.029	0.391 ⟨ 0.047	0.728 ⟨ 0.087
Total ß-Carotene[c]	0.171 ± 0.017	0.319 ⟨ 0.032	0.436 ⟨ 0.034	0.812 ⟨ 0.064
Cholesterol	1335 ± 13	3454 ⟨ 35	1669 ⟨ 17	4318 ⟨ 43

[a] The true concentration of these analytes is expected, with 95 % confidence, to be in the interval defined by the certified value ± the expanded uncertainty. See section on "Certified Values and Their Uncertainties" for computational details.

[b] Includes β-tocopherol.

[c] Includes *cis* and *trans* isomers of β-carotene.

Table 2. Reference Concentration Values for Carotenoids and Vitamin D in SRM 968c[a]

	Level I		Level II	
	µg/mL	µmol/L	µg/mL	µmol/L
trans-Lutein	0.047 ± 0.007	0.08 ± 0.01	0.068 ± 0.007	0.12 ± 0.01
Total Lutein[b]	0.057 ± 0.008	0.10 ± 0.01	0.088 ± 0.009	0.15 ± 0.02
Total Zeaxanthin[b]	0.026 ± 0.009	0.05 ± 0.02	0.019 ± 0.009	0.03 ± 0.02
Total ß-Cryptoxanthin[b]	0.072 ± 0.008	0.13 ± 0.02	0.030 ± 0.006	0.05 ± 0.01
trans-Lycopene	0.13 ± 0.03	0.24 ± 0.05	0.17 ± 0.03	0.32 ± 0.06
Total Lycopene[c]	0.34 ± 0.04	0.63 ± 0.07	0.45 ± 0.07	0.8 ± 0.1
trans-α-Carotene			0.09 ± 0.01	0.17 ± 0.02
Total α-Carotene[b,d]	0.020 ± 0.006	0.04 ± 0.01	0.10 ± 0.02	0.20 ± 0.03
25-Hydroxyvitamin D	0.015 ± 0.002	0.036 ± 0.004	0.016 ± 0.002	0.038 ± 0.004

[a] The true concentration of these analytes is expected, with 95 % confidence, to be in the interval defined by the reference value ± the expanded uncertainty. See section on "Reference Values and Their Uncertainties" for computational details.
[b] May include *cis* and *trans* isomers.
[c] Includes the *cis* and *trans* isomers of lycopene; may include other carotenoid compounds.
[d] For Level I, no *cis* isomers were detected. Therefore, *trans*- and total α-carotene concentrations in this level may be considered to be equivalent.

Table 3. Information Concentration Values for Fat-Soluble Vitamins and Carotenoids in SRM 968c[a]

	Level I		Level II	
	µg/mL	µmol/L	µg/mL	µmol/L
25-Hydroxyvitamin D_2[b]	0.0019	0.0046	0.0028	0.0069
25-Hydroxyvitamin D_3[b]	0.012	0.029	0.012	0.031
trans-2',3'-Anhydrolutein[c]	0.04	0.07	0.05	0.1
Total α-Cryptoxanthin[d]	0.02	0.03	0.02	0.03
Total *cis*-ß-Cryptoxanthin[e]	0.02	0.04	0.02	0.03
Phytofluene[f]	0.05	0.09	0.07	0.1
Retinyl Palmitate[g]	0.03	0.06	0.08	0.2

[a] These are noncertified values with no reported uncertainties as there is insufficient information to assess uncertainties. The information concentration values (except for total α-cryptoxanthin and retinyl palmitate; see footnotes below) were derived from results submitted by only one laboratory.
[b] Concentration is based on absorptivity of 18 300 dL/g•cm in ethanol at 265 nm [21].
[c] Concentration is based on absorptivity of lutein used by the reporting laboratory (2672 dL/g•cm in ethanol at 453 nm) [22].
[d] Concentration value was derived from results reported by six collaborating laboratories.
[e] Concentration is based on absorptivity of ß-cryptoxanthin (2356 dL/g•cm in ethanol at 452 nm) [22].
[f] Concentration is based on absorptivity of 1577 dL/g•cm in hexane at 348 nm [23].
[g] Concentration value is the median of results reported by 13 laboratories for Level I and 15 laboratories for Level II.

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[21] Absorptivity provided by J.A. Beaulieu, DiaSorin, Inc., Stillwater, MN, USA.

[22] Absorptivity and results provided by A.A. Franke and L.J. Custer, Cancer Research Center of Hawaii, University of Hawaii at Manoa, Manoa, HI, USA.

[23] Absorptivity and results provided by E.R. Wiley, U.S. Department of Agriculture, Beltsville, MD, USA.

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[29] Absorptivity for β-cryptoxanthin in ethanol is calculated from the values for β-cryptoxanthin in petroleum ether and β-carotene in ethanol and petroleum ether provided in Reference 28.

Users of this SRM should ensure that the certificate in their possession is current. This can be accomplished by contacting the SRM Program at: (301) 975-6776 (select "Certificates"), Fax (301) 926-4751, e-mail srminfo@nist.gov, or via the Internet http://ts.nist.gov/srm.

APPENDIX A

Analysts at the institutions listed below performed measurements that contributed to the value assignment of constituents in SRM 968c.

American Medical Laboratories, Chantilly, VA, USA
ARUP Laboratories, Salt Lake City, UT, USA
Biochemistry Department, Royal Liverpool Children's Hospital, Liverpool, England
Bio-Reference Laboratories, Elmwood Park, NJ, USA
Cancer Prevention and Control, University of California at San Diego, La Jolla, CA, USA
Cancer Research Center of Hawaii, University of Hawaii at Manoa, Honolulu, HI, USA
Centers for Disease Control and Prevention, Atlanta, GA, USA[a]
Central Laboratory Services, Inc., Pittsburgh, PA, USA
Children's Hospital Medical Center, Seattle, WA, USA
Children's Hospital National Medical Center, Washington, DC, USA
Craft Technologies, Inc., Wilson, NC, USA
Department of Clinical Laboratory Science, State University of New York at Buffalo, Buffalo, NY, USA
Department of Human Nutrition and Dietetics, The University of Illinois at Chicago, Chicago, IL, USA
Department of Laboratory Medicine, University of Washington, Seattle, WA, USA
Department of Laboratory Medicine and Pathology, University of Alberta Hospitals, Alberta, Canada
Department of Medicine, Manchester Royal Infirmary, Manchester, England[b]
Department of Medicine, Michigan State University, East Lansing, MI, USA
Department of Pathology, Parkland Memorial Hospital, Dallas, TX, USA
EMA Medical Laboratory, Inc., Ridgewood, NY, USA
The Endocrine Laboratory, Charing Cross Hospital, London, England[b]
Fred Hutchinson Cancer Research Center, Seattle, WA, USA
Hôpital Universitaire Albert Michallon, La Tronche, France
Hospital Clinic I Provincial de Barcelona, Barcelona, Spain
Human Nutrition Unit, National Institute of Nutrition, Rome, Italy
Immunoassay Laboratory, The Royal Hospital of St. Bartholomew, London, England[b]
Instituto de Investigación Nutricional, Lima, Peru
International Centre for Diarrhoeal Diseases Research, Dhaka, Bangladesh
Laboratoire de Biochimie, Hôpital Purpan, Toulouse, France
Le Directeur des Services Economiques, Paris, France
M.D. Anderson Cancer Center, Houston, TX, USA
MDS Laboratory Services, Etobicoke, Canada[b]
Medical Research Laboratories, Highland Heights, KY, USA
MRC-Resource Centre for Human Nutrition Research, Cambridge, England
Neonatal Research, University of Louisville, Louisville, KY, USA
Nutrition Diet and Health Department, Institute of Food Research, Norfolk, England
Pantox Laboratories, San Diego, CA, USA
Pediatric CRC CORE Laboratory, University of Colorado Health Sciences Center, Denver, CO, USA[a]
Quest Diagnostics, Inc., Baltimore, MD, USA
Quintiles Laboratories, Ltd., Smyrna, GA, USA
Rowett Research Institute, Aberdeen, Scotland
Strong Memorial Hospital, Rochester, NY, USA
Servicio de Nutricion, Clinica Puerta de Hierro, Madrid, Spain
TNO Nutrition and Food Research, Zeist, The Netherlands
Unité de Vitaminologie, Laboratoire Marcel Mérieux, Lyon, France
University of California at Los Angeles School of Medicine, Los Angeles, CA, USA
University of Illinois at Urbana-Champaign, Urbana, IL, USA
University of Maryland Medical System, Baltimore, MD, USA
University of Minnesota, Minneapolis, MN, USA
University of Stellenbosch, Tygerberg Campus, Tygerberg, South Africa
U.S. Department of Agriculture, Beltsville, MD, USA
Vitamin A Laboratory, University of Zimbabwe, Harare, Zimbabwe
Vitamins and Fine Chemical Division, F. Hoffmann-La Roche Ltd., Basel, Switzerland
The Wilmer Eye Institute, The Johns Hopkins University, Baltimore, MD, USA

[a] NIST Micronutrients Measurement Quality Assurance (QA) Program participant who also provided vitamin D data.
[b] Not a participant in the QA program. Provided values for vitamin D only.

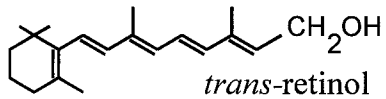
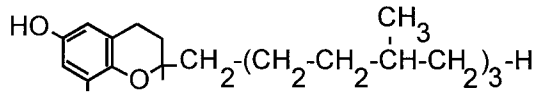
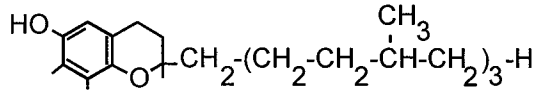
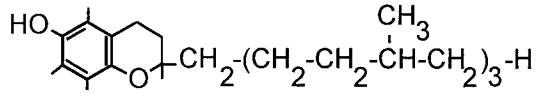
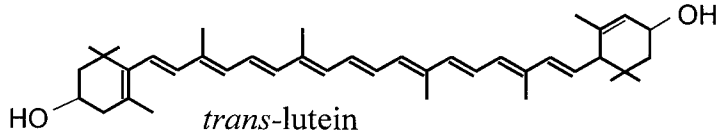
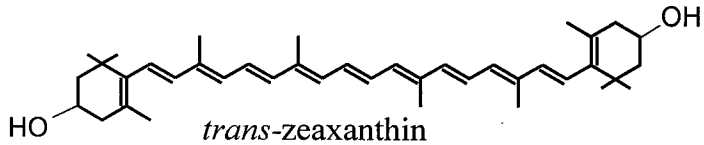
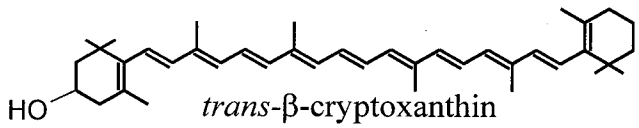
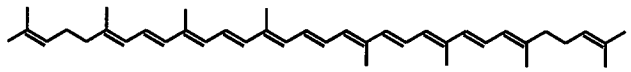
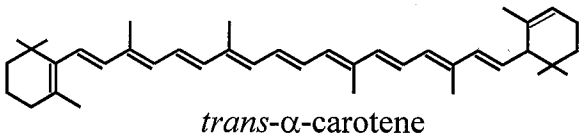
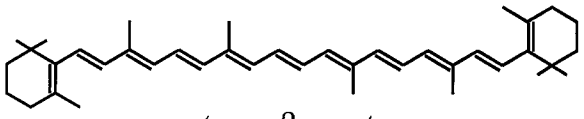
COMPOUND	λ_{max}	ABSORPTIVITY
 *trans*-retinol	325 nm	1843 dL/g·cm in ethanol
 δ-tocopherol	297 nm	91.2 dL/g·cm in ethanol
 γ-tocopherol	298 nm	91.4 dL/g·cm in ethanol
 α-tocopherol	292 nm	75.8 dL/g·cm in ethanol
 *trans*-lutein	445 nm	2550 dL/g·cm in ethanol
 *trans*-zeaxanthin	452 nm	2540 dL/g·cm in ethanol
 *trans*-β-cryptoxanthin	452 nm	2356 dL/g·cm in ethanol
 *trans*-lycopene	472 nm	3450 dL/g·cm in hexane
 *trans*-α-carotene	444 nm	2800 dL/g·cm in hexane
 *trans*-β-carotene	452 nm	2592 dL/g·cm in hexane

Figure 1. Wavelength maxima and absorptivites used for calibration at NIST [24-29].

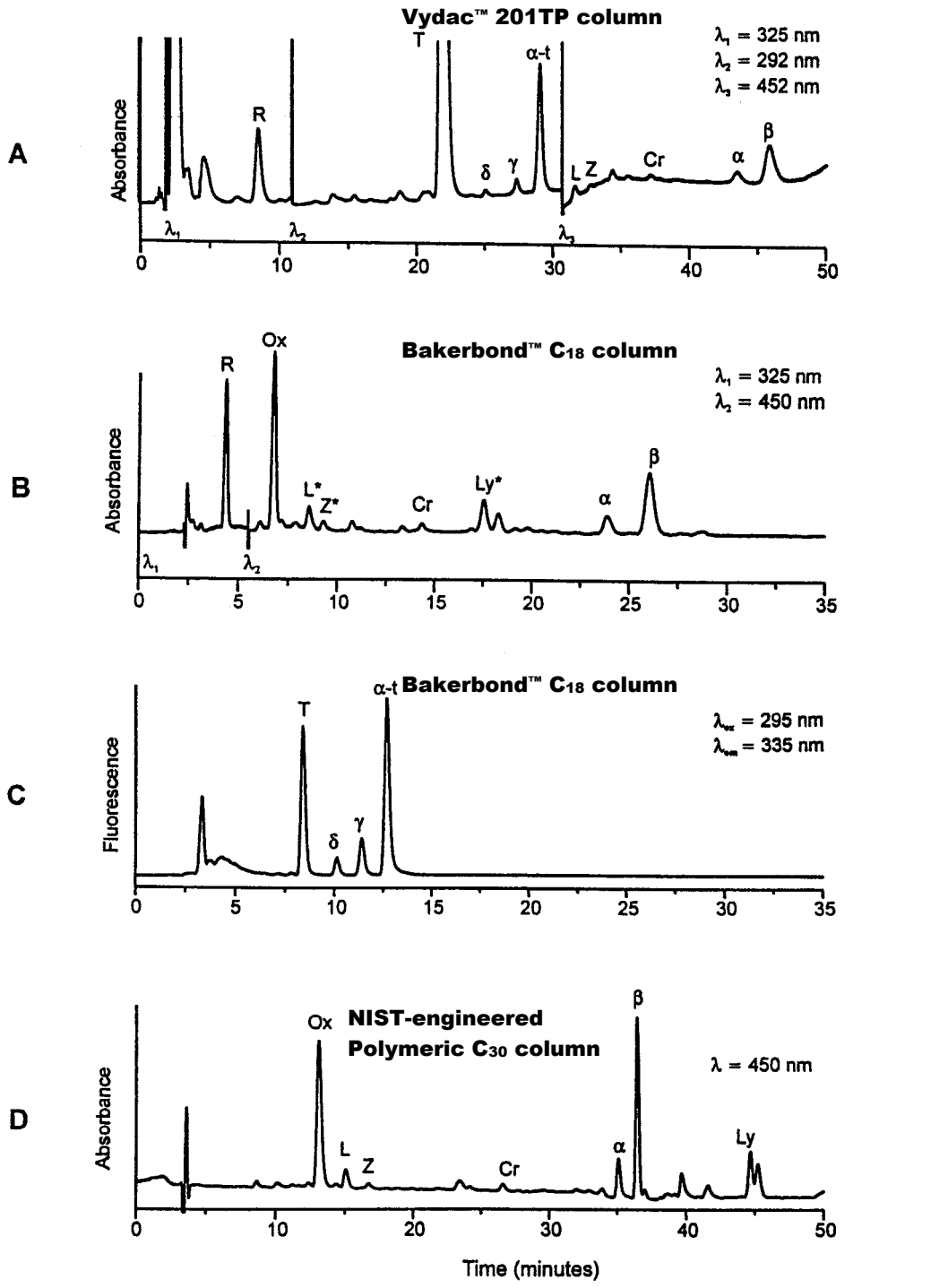


Figure 2. Chromatograms from the analyses of Level II of SRM 968c using three different LC methods performed at NIST. Chromatographic conditions are described in the text.